PB82-124264

A Method for the Determination of Dialkyl Phosphate Residues in Urine

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

Sep 81

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TECHNICAL REPORT DATA

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1. REPORT NO. EPA-600/J-81-151	2. JOURNAL ARTICLE
3. PB82-124264	
4. TITLE AND SUBTITLE: A METHOD FOR THE DETERMINATION OF DIALKYL PHOSPHATE RESIDUES IN URINE	5. REPORT DATE: September 1981
	6. PERFORMING ORGANIZATION CODE: 306056
7. AUTHOR(S): Stuart J. Reid and Randall R. Watts	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Environmental Toxicology Division, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711	10. PROGRAM ELEMENT NO.: ABZE1A
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS: Office of Research and Development (RTP,NC), Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, North Carolina 27711	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE: EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Journal of Analytical Toxicology 5: 126-132, 1981

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY NATIONAL TECHNICAL INFORMATION SERVICE U.S. DEPARTMENT OF COMMERCE SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT: RELEASE TO PUBLIC	19. SECURITY CLASS (This Report): UNCLASSIFIED	21. NO. OF PAGES
	20. SECURITY CLASS (This page): UNCLASSIFIED	22. PRICE

Journal of Analytical Toxicology, Vol. 5, May/June 1981

EPA-600/J-81-151
Journal Article

A Method for the Determination of Dialkyl Phosphate Residues in Urine*

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Abstract

The analysis of urine for dialkyl phosphate residues provides a measure of mammalian exposure to organophosphate pesticides. A method is presented for quantitative determination of six alkyl phosphate urinary metabolites. These metabolites are as follows: O,O-dimethyl phosphate, O,O-diethyl phosphate, O,O-dimethyl phosphorothionate, O,O-dimethyl phosphorodithioate, O,O-diethyl phosphorthionate, and O,O-diethyl phosphorodithioate. A screening method is also given for a rapid assessment of human exposure. Pentafluorobenzyl bromide is utilized as the derivatization reagent to form pentafluorobenzyl esters. The reaction products are determined by gas chromatography on routine pesticide columns utilizing the phosphorus specific flame photometric detector. Recoveries of all six dialkyl phosphates are greater than 90% with the minimum level of detection ranging from 0.04 to 0.13 ppm.

Introduction

The widespread use of the non-persistent, acutely toxic organophosphate pesticides has created a need for a fast and accurate analytical method for the estimation of human exposure. Depression of blood cholinesterase levels has been used as a measure of exposure, but has been found to be of low sensitivity (1). The analysis of dialkyl phosphates in urine provides a more accurate and sensitive assessment of exposure. Residue levels in urine have also been found to be dose-dependent (2).

St. John and Lisk (3) converted the dialkyl phosphate residues in urine to a volatile methyl ester derivative before gas chromatographic determination. Shafik *et al.* (4) used diazopentane to derivatize the dialkyl phosphates after solvent extraction from urine. A method using an ion exchange resin to isolate the dialkyl phosphate metabolites from urine was introduced by Lores and Bradway (5). These procedures have enabled field laboratories to monitor the alkyl phosphate degradation products of organophosphate pesticides in urine. However, a review of analytical results for urine quality assurance samples from such laboratories indicates considerable method improvement is needed to significantly increase both precision and accuracy. A recent investigation by Ito *et al.* (6) corroborates this conclusion. A series of ten analyses utilizing diazopentante as the derivatizing reagent yielded a coeffiecient of variation for diethyl thiophosphate (DETP) of 41%. In addition, the diazoalkane reagents employed in these methods (3-5) are carcinogenic and explosive.

The use of triazenes as the alkylating agent in the derivatization of the dialkyl phosphates obtained from urine has been reported, but the recoveries were not given (7,8). Churchill *et al.* (9) used alcoholic trimethyl phenyl ammonium hydroxide as a gas chromatography co-injection reagent to achieve in-block methylation of alkyl phosphate standards containing sulfur. However, the procedure was not demonstrated as effective for derivatization of dimethyl phosphate (DMP) and diethyl phosphate (DEP). Kawahara (10) reported the use of pentafluorobenzyl bromide in the derivatization of phenols and mercaptans.

This paper describes a new rapid screening method and a more rigorous quantitative method for the determination of six dialkyl phosphate residues in urine. The urine is subjected to azeotropic distillation, followed by derivatization with pentafluorobenzyl bromide to form the pentafluorobenzyl esters of the dialkyl phosphates. This procedure permits only single reaction products with the dialkyl thiophosphates. Results are, therefore, not complicated by production of both possible isomers. Gas chromatography with phosphorus specific flame photometric detection (FPD) is used as the determinative step. Recoveries from fortified urine of over 90% were obtained for all six dialkyl phosphates.

*Presented in preliminary form at the 31st Annual Southeastern Regional Meeting of the American Chemical Society, October 24-26, 1979, Roanoke, VA.

**This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Experimental

Reagents

Pentafluorobenzyl bromide (PFB-Br) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). *Caution:* PFB-Br is a potent lachrymator. The acetone, hexane, acetonitrile, methanol, and benzene were pesticide quality solvents.

Silica gel, activity grade I, Woelm, ICN Nutritional Biochemicals (Cleveland, Ohio) was activated at 175°C for 24 hours. Deactivated silica gel was prepared from activated grade I by placing 5.0 g in a tube with a Teflon-lined screw cap, adding 1.0 mL of water, and rotating in a mixer for 2 hours at 50 rpm.

Sodium sulfate, granular, anhydrous, was purified by Soxhlet extraction with methylene chloride by 50 cycles.

Standards

The dialkyl phosphates, their pentafluorobenzyl (PFB) derivatives, and abbreviations are listed in Table I.

The dialkyl thiophosphate standards were kindly supplied by American Cyanamid (Princeton, New Jersey). The dialkyl phosphate compounds were supplied by the EPA Pesticides Repository (Research Triangle Park, North Carolina).

The 0.80 μg/mL working standard solutions were prepared from 200 μg/mL methanol stock solutions. The working solution containing the dialkyl thiophosphates, dimethyl phosphorothionate (DMTP), dimethyl phosphorodithioate (DMDTP), diethyl phosphorothionate (DETP), and diethyl phosphorodithioate (DEDTP), was prepared with acetonitrile for Method I and with acetone for Method II. The working solution for the alkyl phosphates, DMP and DEP, as well as the standard solutions used for spiking urine were prepared with acetonitrile.

Apparatus

A Tracor Model 222 gas chromatography (GC) (Austin, Texas) equipped with a flame photometric detector (FPD) was operated in the phosphorus mode at 526 nm. GC columns were 1.8 m x 4 mm i.d. borosilicate glass packed with 5% OV-210, 1.5% OV-17/1.95% OV-210 or 4% SE-30/6% OV-210 all coated with Gas Chrom Q, 80/100 mesh. The operating conditions were as follows: column oven, 183°C, inlet, 215°C; detector, 175°C; nitrogen carrier gas, 60 mL/min; hydrogen, 50 mL/min; air, 100 mL/min.

Electron impact mass spectra were obtained on a Hewlett-Packard 5930A mass spectrometer (Avondale, Pennsylvania) equipped with a Model 5700A gas chromatograph and a Model 5933A data system. Gas chromatography column and parameters were as follow: 1.5% OV-17/1.95% OV-210 on Gas-Chrom Q, 80/100 mesh; column temperature, 190°C; helium flow rate, 42 mL/min. Mass range was scanned from 0 to 320 amu.

Chromaflex columns, 7 mm Kontes (Vineland, New Jersey), were used for the silica gel chromatography.

Centrifuge tubes, conical, 12 mL, Pyrex, graduated with matching plastic screw caps and Teflon liners from Scientific Products (McGraw Park, Illinois) were used for the derivatization reactions. *Caution:* The tubes and caps were tested for a proper seal by adding 1 mL of acetonitrile, capping, and heating for one hour at 90°C. Tubes that gave a poor seal were not used.

Concentration of solutions were carried out with a nitrogen evaporator and water bath, Organomation Associates (Worceter, Massachusetts).

Both the azeotropic distillation of the urine samples and the 90°C derivatizations were performed in a tube heater from Kontes Glass Co. (Vineland, New Jersey) or a bath/circulator, Brinkman Instrument (Westbury, New York), containing propylene glycol, laboratory grade, from Fischer Scientific Co. (Fairlane, New Jersey).

A vortex mixer from Scientific Industries (Springfield, Massachusetts) and a Model HN-S centrifuge from International Equipment Co. (Needham Height, Massachusetts) was used.

A 0-100 μL calibrated microdispenser from Drummond Scientific Company (Broomall, Pennsylvania) was employed for dispensing PFB-Br reagent.

Procedure

Two methods are described. Method I is a rapid screening procedure that is recommended for determination of mammalian exposure to total organophosphate pesticides. Method II is a quantitative procedure for determination of each of the six alkyl phosphate residues after separation into thio and non-thio fractions.

Table I. Dialkyl Phosphates and PFB Derivatives.

Abbreviations	Dialkyl Phosphates	Formula	Derivative
DMP	0,0-Dimethyl phosphate	$(CH_3O)_2$ P(=O)-OH	0,0-Dimethyl O-PFB phosphate
DEP	0,0-Diethyl phosphate	$(C_2H_5O)_2$ P(=O)-OH	0,0-Diethyl O-PFB phosphate
DMTP	0,0-Dimethyl phosphorothionate	$(CH_3O)_2$ P(=S)-OH	0,0-Dimethyl S-PFB phosphorothiolate
DMDTP	0,0-Dimethyl phosphorodithioate	$(CH_3O)_2$ P(=S)-SH	0,0-Dimethyl S-PFB phosphorodithioate
DETP	0,0-Diethyl phosphorothionate	$(C_2H_5O)_2$ P(=S)-OH	0,0-Diethyl S-PFB phosphorothiolate
DEDTP	0,0-Diethyl phosphorodithioate	$(C_2H_5O)_2$ P(=S)-SH	0,0-Diethyl S-PFB phosphorodithioate

Method I

A 1.0 mL sample of urine was added to a 12 mL screw-top centrifuge tube containing 7 mL of acetonitrile. After brief vortex mixing, the tubes were centrifuged at 2500 rpm. The supernate containing the extracted alkyl phosphate salts was decanted into another 12 mL centrifuge tube. A few carborundum chips were added and water was removed by azeotropic distillation in a 80°C bath. The solution was concentrated to about 0.3 mL. Residual solvent was removed by evaporation to dryness in a 35°C bath under a gentle stream of nitrogen.

Acetonitrile (1.0 mL) was added to the sample residue followed by 50 µL PFB-Br reagent and about 35 mg of anhydrous potassium carbonate. A 1.0 mL standard solution of DMTP, DMDTP, DETP, and DEDTP and a separate 1.0 mL standard solution of DMP and DEP were treated similarly.

Sample and standard tubes were capped with Teflon lined screw caps and placed in a 90°C bath for two hours. Aliquots of the cooled supernate were analyzed by GC/FPD for PFB derivatives of all six dialkyl phosphates.

Method II

The method described here is also outlined in Figure 1.

A 1.0 mL sample of urine was added to a 12 mL screw-top centrifuge tube containing 7 mL of acetonitrile. After brief vortex mixing, the tubes were centrifuged at 2500 rpm. The supernate, containing the extracted alkyl phosphate salts, was decanted into another 12 mL centrifuge tube. A few carborundum chips were added and water was removed by azeotropic distillation in a 80°C bath. The solution was concentrated to about 0.3 mL. Residual solvent was removed by evaporation to dryness in a 35°C bath under a gentle stream of nitrogen.

Thio Alkyl Phosphates: One milliliter of acetone was added to the sample residue followed by 50 µL of PFB-Br. To separate tubes containing a 1.0 mL acetone standard solution of DMTP, DMDTP, DETP, and DEDTP, was also added 50 µL of PFB-Br. Sample and standard tubes were capped and reacted at room temperature for 1 hour. Aliquots of 1-10 µL were analyzed by GC/FPD for quantitation of sulfur-containing metabolites. Further silica gel cleanup may also be effected on the hexane extract of the derivatized thio phosphates as described later.

Non-Thioalkyl Phosphates: The acetone was *carefully* removed from the sample tubes by nitrogen evaporation in a room temperature bath until about 50 µL of acetone and/or reagent remained. To the residue was added 5 mL of hexane and about 10 mg anhydrous potassium carbonate. The tubes were mixed on the vortex mixer and centrifuged at 2500 rpm for 5 minutes. The supernate containing derivatized thiophosphates was decanted with care (to avoid transfer of solids) into a separate tube for subsequent silica gel separation if necessary. The centrifugate containing unreacted DMP and DEP was evaporated to dryness under nitrogen in a 35°C bath.

To the sample residue was added 1.0 mL of acetronitrile, followed by 50 µL of PFB-Br reagent and about 25 mg of anhydrous potassium carbonate. To a 1.0 mL acetonitrile standard solution of DMP and DEP in a separate tube, was added 50 µL PFB-Br and 25 mg anhydrous potassium carbonate. Capped samples and standards were reacted together in a bath at 90°C for 2 hours. Aliquots of the cooled supernates were analyzed by GC/FPD for quantitation of the derivatized DMP an DEP.

Fortified urine samples were prepared by addition of an acetonitrile solution of dialkyl phosphates just prior to the acetonitrile azeotropic distillation.

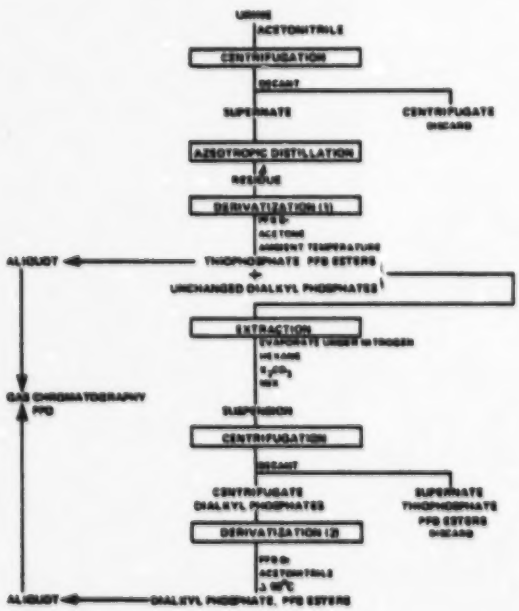

Figure 1. Method for the determination of dialkyl phosphates in urine. (Method II).

Silica Gel Chromatography

A 7 mm i.d. chromaflex column was prepared by first adding a small glass-wool plug followed by 1.0 g of deactivated silica gel and topped with 25 mm of anhydrous sodium sulfate. The column was washed with 10 mL of hexane and the eluate discarded. A 1 mL hexane solution containing the derivatized thioalkyl phosphate was transferred to the column. An additional 9 mL of hexane was eluted through the column and discarded (fraction I). The column was then eluted with 10 mL of benzene (fraction II) and 10 mL of acetone (fraction III).

Caution: Benzene is a suspect carcinogen. Use only in the hood. Avoid skin contact.

Fraction II contains the PFB esters of the dithiophosphates (DMDTP and DEDTP). Fraction III contains the PFB esters of the monothiophosphates (DMTP and DETP).

The collected fractions were concentrated to 1.0 mL under a gentle stream of nitrogen in a 35°C bath. Aliquots were then injected into the GC/FPD for quantitation of the derivatized dialkyl thiophosphates.

Mass Spectra

A mass spectrum was obtained for the PFB esters of DMP after silica gel cleanup.

Results and Discussion

Method Development

The alkylation of the dialkyl thiophosphates is effected at ambient or elevated temperatures, while the alkylation of DMP and DEP proceeds in good yield only at elevated temperatures. Unfortunately, the thio compounds decompose somewhat with heating to their respective non-thio forms of DMP and DEP. An approximate 25% decomposition has been observed for DMTP while only 10% decomposition has been evidenced with DETP. The dialkyl thiophosphates also show decomposition, but to a considerably lesser extent than their respective monothio forms. For an accurate analysis of the individual alkyl phosphate salts, it is therefore necessary to separate the dialkyl thiophosphates from the DMP and DEP before the 90°C derivatization of the latter. Toward this end, the four thiophosphates were derivatized at room temperature (Method II). This reduces the polarity of the thio compounds and renders them hexane soluble. The dialkyl phosphates, DMP and DEP, do not react at room temperature and are insoluble in hexane. They are separated by centrifugation from a hexane extract and then derivatized in acetonitrile solution at 90°C.

The Method I screening procedure is recommended for an expenditious determination of alkyl phosphate salts in urine. A rapid analysis is likely to be more important than introduction of a small error in the determination of the relative amounts of DMTP and DMP. As previously mentioned, significant error will occur only if DMPT is present and will manifest itself as an increased amount of DMP. Total residues, of course, would not be affected.

Both acetonitrile and isopropyl alcohol proved to be effective in the removal of water through azeotropic distillation of urine. However, acetonitrile was more efficient with a 4°C lower boiling point (76°C *vs* 80°C) and 4% higher water content (16% *vs* 12%) in the distillate of the constant boiling mixture.

After room temperature derivatization of the thiophosphates (Method II), care should be exercised that evaporation of the acetone solution under nitrogen does not proceed to total dryness. A small amount of the PFB-Br reagent should remain. Some DMTP has been found in the 90°C derivatization mixture when the room temperature reaction residue goes to complete dryness. This finding indicates that the DMTP-PFB derivative may revert back to free DMTP upon depletion of the PFB-Br. The free DMTP formed would not be soluble in the subsequent hexane extraction and would, therefore, appear with DMP and DEP in the later derivatization. This also creates the potential for thermal decomposition of any DMTP that is carried over to give an elevated recovery of DMP.

The Method II ambient temperature alkylation of the thio compounds proceeds rapidly without the necessity of an alkaline calayst. However, the 90°C derivatization of the dialkyl phosphate requires anhydrous potassium carbonate for adequate reaction efficiency. Potassium hydroxide, ammonium hydroxide, sodium bicarbonate, several quaternary ammonium salts and tertiary amines were tried, but recoveries were lower than with potassium carbonate. The tertiary amines gave lower recoveries, possibly because of competing debenzylation reactions of the amine with the trialkyl phosphate or quaternary ammonium salt formation of the amine with PFB-Br.

DMP and DEP do not react with PFB-Br and potassium carbonate at 90°C in hexane or toluene. The reaction does take place in acetone, but not as effectively as in acetonitrile. It seems that the more polar the solvent, the better the recovery. However, the reaction goes poorly in the protic solvent methanol. The less polar isopropyl alcohol allows an efficient reaction.

Room temperature reactions of the thio compounds with PFB-Br do not proceed in hexane, only at a slow rate in toluene or methylene chloride, but rapidly in acetone, acetonitrile, and methanol.

Structure of the Derivative

Both the diazoalkane (1) and the triazene alkylation reactions (7) of DMTP and DETP produce two isomers of each metabolite, the thiono and the thiolo esters. The dialkyl thiophosphoric acids are in tautomeric equilibrium and thus give mixed products with alkylating agents (11). This made quantitation of these compounds difficult as the ratio of the two isomers might have changed with a change in reaction conditions. The PFB-Br alkylation procedure reported in this paper yields only one product each for DMTP and DETP. Since the reaction occurs with the ionized species, the more nucleophilic sulfur atom would be expected to react with an electrophilic agent to preferentially form the thiolate ester. Kabachnik (11) found that the reaction of the sodium salt of DETP with methyl iodide produces only O,O-diethyl-S-methyl phosphorothiolate. Earlier, it was determined that treatment of DETP potassium salt with bromoacetronitrile or chloacetyle urea resulted in the formation of the only thiolate compounds (12). These reports (11,12) therefore, suggest that the thiolate pentafluorobenzyl esters are the expected products resulting from the derivatizations reported here.

The mass spectrum of the dimethyl pentafluorobenzyl ester is presented in Figure 2. The expected breakdown products and the molecular ion m/z-306 confirm the proposed structure.

Reaction Mechanism

The rate of reaction of DMP with PFB-Br was determined at DMP concentrations of 0.1 ng/μL, 1 ng/μL and 10 ng/μL. The response was found to be a linear function ($r = 0.999$) of the concentration of DMP, thus, indicating the rate is independent of the DMP concentration.

The increase of DMP-PFB with time in the derivatization reaction appears linear (Figure 3) with a correlation coefficient of 0.996, indicating a zero order reaction. Since the PFB-Br reagent is in excess, the actual reaction would be first order. The suggested S_N1 mechanism is a nucleophilic reaction of the dialkyl phosphate anion with the PFB cation (Figure 4). The formation of the resonance stabilized PFB carbonium ion (step 1) is the rate-limiting step.

Gas Chromatography

The responses of the six dialkyl phosphates were determined from 0.1 to 60 ng. All compounds were found to be linear ($r > 0.990$) in this range.

Figure 5 shows the GC separation of the four derivatized dialkyl thiophosphates. A partial separation of all the compounds was obtained on a 4% SE-30/6% OV-210 column. However, this separation was not achieved on another SE-30/OV-210 column of similar theoretical plates

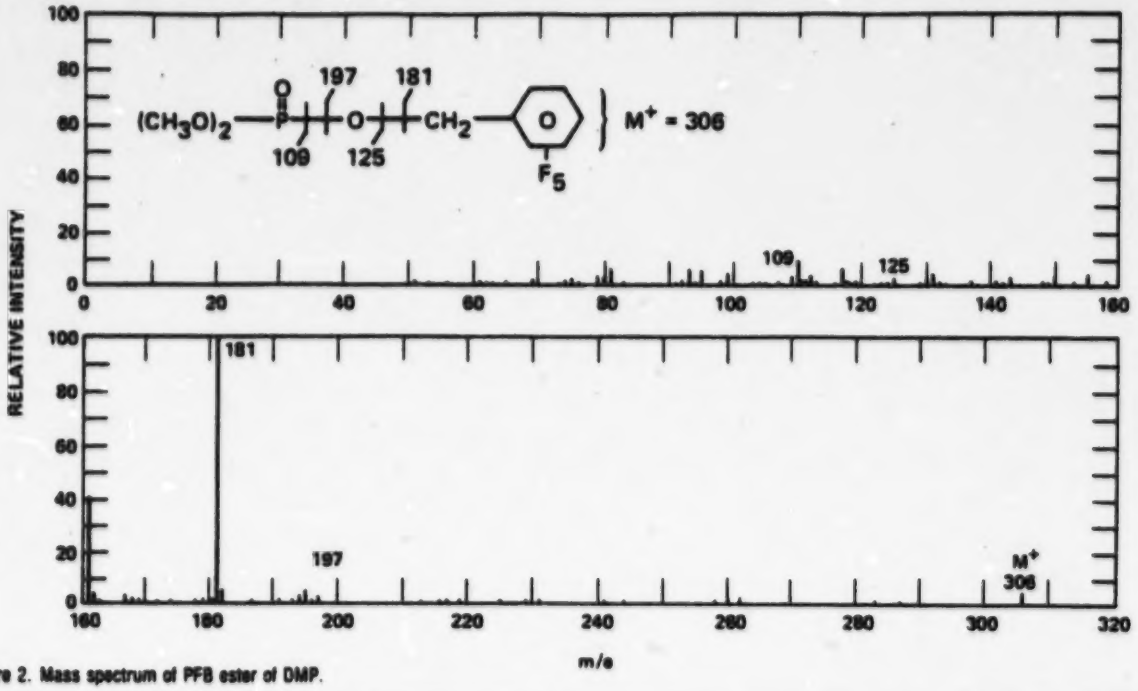

Figure 2. Mass spectrum of PFB ester of DMP.

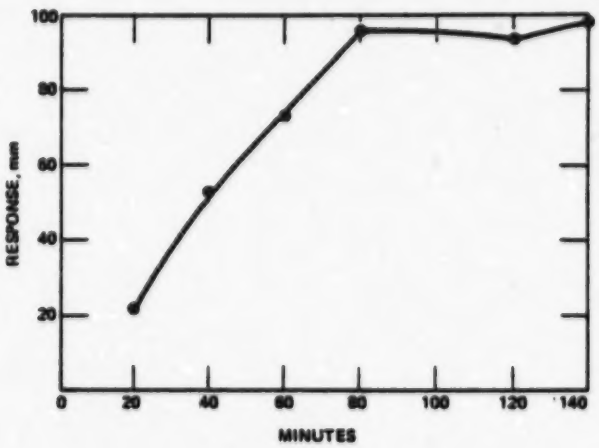

Figure 3. Time course of the reaction of DMP with PFB-Br.

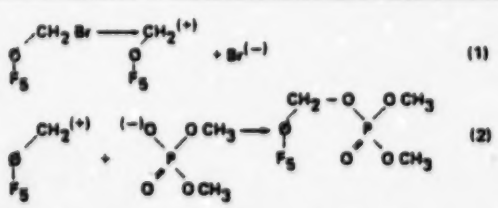

Figure 4. Suggested S_n1 mechanism for the reaction of PFB-Br with DMP.

and chromatographic conditions. Work is continuing to resolve this. Domínguez *et al.* (13) have found that mixed stationary phases undergo selective loss of the most volatile component on aging. This results in changes in the retention times with concomitant changes in the resolution of sample components in gas chromatography. Good GC separation of the dialkyl phosphates was obtained, as indicated in Figure 6.

If DMTP is not resolved from DMDTP, and DETP is not resolved from DEDTP on the GC column, then passage through silica gel will provide a fraction II containing DMDTP and DEDTP and a fraction III containing DMTP and DEPT. The components of each fraction are easily resolved as is apparent from the retention times in Table II, a list of the retention times of the dialkyl phosphate derivatives. It appears that retention time is a function of molecular weight in this group of compounds.

The structure of the derivative makes several detection means possible in addition to flame photometric detection. The halogens in the ring makes electron capture (EC) a sensitive means of detection. Standard DMP-PFB gave a minimum level of EC detection of 0.8 ppb. However, urinary sample derivatives must be further purified before EC can be used. Similarly, the nitrogen/phosphorous (NP) and electrolytic conductivity detectors can be used with purified urinary metabolite derivatives. The aromatic ring also makes UV detection in high performance liquid chromatography (HPLC) a possibility.

Reproducibility Accuracy and Sensitivity of the Method

Derivative standards prepared by first evaporation of the standard solution to dryness under nitrogen and then derivatization with PFB-Br solution showed less reproducibility than

Figure 5. Chromatograms of PFB esters of dialkyl thiophosphates using a 4% SE-30/6% OV-210 column with flame photometric detection: 1.6 ng each: (A) standards; (B) extract from spiked urine.

Table II. Relative Retention Times of PFB Esters of Dialkyl Phosphates on Three Columns.

Dialkyl Phosphate	4% SE-30/ 6% OV-210	5% OV-210	1.5% OV-17/ 1.95% OV-210
DMP	1.00	1.00	1.00
DEP	1.35	1.17	1.14
DMTP	1.61	1.28	2.07
DMDTP	1.70	1.28	2.07
DETP	2.09	1.91	2.50
DEDTP	2.22	1.91	2.50

Column temperatures were 183°C; carrier gas flow rates were 60 ml/min.

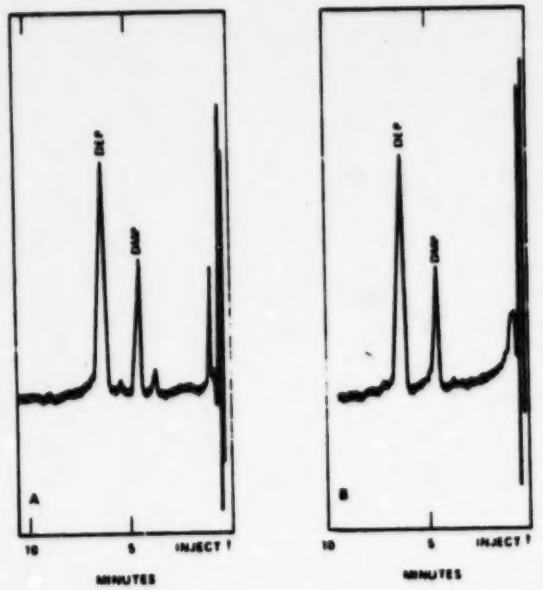

Figure 6. Chromatograms of PFB esters of dialkyl phosphates using a 4% SE-30/6% OV-210 column with flame photometric detection; 1.6 ng each: (A) standards; (B) extract from spiked urine.

those prepared by adding the reagent to a standard solution at the time of derivatization of samples. The alternative of making up the standard from a concentrate, adding reagents, and diluting to volume has the disadvantage that the standard is exposed to the reagent for an indefinite period of time.

The accuracy and precision of the method is indicated in Table III. This data is derived from five determinations. The minimum level of detection is also listed in Table III.

Acknowlegement

The authors wish to thank Dr. Wayne Sovocool for obtaining the mass spectra.

Table III. Recovery and Detection Limits of Dialkyl Phosphates* from Urine.

	Recovery, %	SD	CV	Minimum Level of Detection** ppm
DMP	91	2.3	5.4	0.13
DEP	96	3.1	4.7	0.072
DMTP	92	6.4	6.9	0.052
DMDTP	98	0.87	0.88	0.038
DETP	99	2.3	2.4	0.041
DEDTP	102	4.0	4.0	0.044

*The concentration was 0.8 ppm.
**A signal 2 x the noise level.

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Manuscript received March 27, 1981.